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ORIGINAL ARTICLE

Biochemical and molecular analysis of *Camellia sinensis* (L.) O. Kuntze tea from the selected P/11/15 clone

Samynathan Ramkumar [a,*], Perisamy Suresh kumar [b], Gandhi Sudhakar [b], J. Anitha [a], Sanmugam Geetha [c], Padmanabhan Mohankumar [d], Velliyur Kanniappan Gopalakrishnan [a]

[a] *Department of Biochemistry and Bioinformatics, Karpagam University, Coimbatore 641021, TN, India*
[b] *Department of Biotechnology, Anna University, BIT Campus, Tiruchirappalli, TN 620024, India*
[c] *Department of Plant Biotechnology, Centre for Plant Molecular Biology, TNAU, Coimbatore 641003, India*
[d] *UPASI Tea Research Foundation, UPASI Tea Research Institute, Valparai 642 127, Coimbatore District, TN, India*

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Gene expression analysis

Abstract Green tea is one of the most important beverages consumed across the world and it possesses various phytotherapeutics. Polyphenol oxidase (PPO) activity, total polyphenols, catechins, amino acid content and enzymatic antioxidants are considered to be potential parameters in tea characterization. P/11/15 clone (*Camellia sinensis* (L) O. Kuntze) was chosen to analyze the biochemical characterization and to analyze the gene expression pattern. The selected P/11/15 clone (*C. sinensis* (L) O. Kuntze) possess potent Polyphenol oxidase (49.62 U/mg of protein), sufficient catechin (20.75%), Polyphenol (20.01%), Peroxidase (450.08 μM of O_2 formed min^{-1} g^{-1} dry weight), Catalase (1.20 μM H_2O_2 reduced min^{-1} mg^{-1} protein) and Super Oxide Dismutase (45.11 U/mg proteins). Flavonoid gene expression reveals ANR (1.66%) and F3H (1.02%) were up regulated in the selected P/11/15 clone. The results obtained suggest that P/11/15 clone showed adequate enzyme levels, thus an increased antioxidant activity.

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1. Introduction

The tea crop shoots (apical bud and two terminal leaves) are harvested for tea manufacturing [1]. Two types of tea are manufactured in India *viz*., CTC (crush, tear and curl) and orthodox depending time of the fermentation. The nature of plucked tea leaves decides the biochemical characteristic which in turn influences the quality of the black tea [2]. The tea crop shoots possess many biochemical constituents, namely, phenolic components, alkaloids, vitamins, enzymes, crude fiber, proteins, lipids and carbohydrates [3,4]. Natural substances, which are presented in plants, help to treat a

* Corresponding author. Tel./fax: +91 422 6611462.
E-mail address: ramkumarbiochem007@gmail.com (R. Samynathan).
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variety of human diseases [5–10]. The black tea possesses desired liquor characteristics and aroma constituents. High quality and aroma enrichment mainly depend on tea leaf constitutes like catechins, chlorophylls, carotenoids, theaflavins, caffeine, and linalool geraniol. Polyphenol oxidase (PPO) peroxidase (PO), phenylalanine ammonia lyase (PAL), chlorophyllase; lipoxygenase and peptidase are present in the shoots which are responsible for the quality and flavor of the tea [11].

The plant's protective system is composed of antioxidant such as peroxidase and catalase [12]. Catalase is the primary H_2O_2 scavenger in the peroxisomes and mitochondria [13]. Peroxidase during tea processing is used to oxidize theaflavins into thearubigins in the presence of hydrogen peroxide. Superoxide dismutase (SOD) is a metalloprotein that catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen. Phenylalanine ammonia lyase (PAL) catalyzes the non-oxidative reaction of L-phenylalanine to trans cinnamic acid. PAL is important in the biosynthesis of the flavanols since it acts as the prime substrate for PPO. Amino acids play an important role in determining the flavor of black tea [14].

Polyphenol oxidase (PPO) is a copper-containing metalloprotein, which is characterized in several higher plant species. It is predominantly located in the thylakoid membranes of chloroplast [15]. PPO oxidizes monophenolic and *o*-diphenol compounds by two types of reactions. First reaction involves the hydroxylation of a monophenol to give a diphenol and second involves the removal of hydrogen from diphenol to give quinine [16]. PPO activity is necessary for black tea fermentation, resulting in the formation of brown tea pigments. The pigments of black tea have been classified into orange colored theaflavins (TF) and brownish thearubigins (TR) based on the oxidation of catechins to *o*-di-Quinones by PPO [17].

Molecular and biochemical marker linked tools are now widely used for screening of tea germplasm. These are believed to be more reliable for identification when compared to conventional phenotypic assessment of genotypes. PPO activity, total polyphenols, catechins and aminoacid contents are considered to be potential parameters in tea characterization [18]. Analyzing the expression patterns of genes is involved in catechin biosynthetic metabolism by reverse transcription-polymerase chain reaction (RT-PCR). Transcript levels of phenylalanine ammonia-lyase 1 (PAL1), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin reductase (LCR) were reported abundant in the young tea leaves. Transcripts of tea homologs of F3H, DFR and LCR, are involved in the high-level accumulation of catechins. A high expression of, F3H and F35H was observed in young and developing leaves [19].

P/11/15 clone of *Camellia sinensis* (L) O. Kuntze is selected for the present study from south Indian estates selection tea accession was to evaluate the biochemical characteristics, quality important enzymes, HPLC fraction analysis of catechin and phenylpropanoid and flavonoid related and level of expression from the crop, shoot (bud, first Internode, first Leaf and, second Leaf) consisting of an apical bud and two terminal leaves.

2. Material and methods

2.1. Plant material collection and extract preparation

P/11/15 clone crop shoots of *C. sinensis* (L) O. Kuntze (apical bud and two terminal leaves) maintained in the uniform age (~10 years old) at the height of 26″ (~60 cm) above ground level were selected. Young tea leaves were collected during morning hour from 8.30 am to 9.00 am. Crop shoots (100 mg) were ground well with 5 ml of ethyl alcohol and made up to 50 ml for biochemical and molecular analysis.

2.2. Biochemical analysis

PPO was estimated from freshly collected crop shoots by the method of Singh and Ravindranath [20]. Bound and soluble form of PPO was estimated from total PPO. The activity was expressed in U/mg of protein. Protein content was determined according to the coomassie blue binding method.

Total polyphenol content was determined using UV–Vis spectrophotometer (Thermo scientific, U.S.A) was adopted the method reported by Dev Choudhury and Goswami [21] and expressed in Gallic acid equivalents. Catechin was quantified by the method of Swain and Hillis [22] and the results were expressed in (+) catechin equivalents.

2.3. Assay for peroxidase

Determination of peroxidase activity was carried out by the following method described by Chance and Maehly [23]. Peroxidase activity was estimated spectrophotometrically at 430 nm and expressed in μmol of O_2 formed min^{-1} g^{-1} dry weight. One unit of peroxidase will form 1 mg of purpurogallin from pyrogallol in 20 s.

2.4. Assay of catalase

Catalase activity was determined following the method of Luck et al. [24]. The activity of catalase was determined spectrophotometrically at 420 nm and expressed as μmol of H_2O_2 reduced min^{-1} mg^{-1} protein.

2.5. Assay of Super Oxide Dismutase (SOD)

SOD activity was estimated by following the modified method of Van Rossun et al. [25]. The assay reaction was carried out at 25 °C and under light and dark conditions. The increase in absorbance at 620 nm due to the blue color formation by NBT photo reduction was measured. The reaction mixtures without tissue supernatant were used as control. The SOD activity was expressed as U/mg of protein. One unit of SOD is defined as the amount of protein that reduces 50% inhibition of NBT reduction.

The segregated two leaves and the bud (5 g each) were homogenized with 80% ice cold aqueous acetone and the suspension was filtered on sintered funnel (Grade-1). The residue was washed repeatedly with ice cold acetone until the powder become colorless. PAL activity was determined using 0.05 M

of phenylalanine as substrate by following the modified methods of Jain [26]. Absorbance was read at 273 nm using UV–Vis spectrophotometer and one unit of enzyme activity is expressed as μmol of cinnamic acid formed per minute per gram of acetone powder.

2.6. Analysis of protein profile (by SDS–PAGE)

The molecular weight of the partially purified PPO was estimated by SDS–PAGE electrophoresis according to the method of Laemmli [27]. The protein was analyzed by SDS–PAGE using 12% polyacrylamide gel, and visualized with Coomassie Blue R-250 staining and its molecular weights were determined by comparing with the molecular weight markers and Genetool, Syngene: (V.4.01).

2.7. Catechin fraction analysis (by HPLC)

Catechin quantification was done by the described method of Swain and Hillis [22]. Oven dried leaf powder (0.2 g) was extracted using 10.0 ml of 70% methanol on a water bath maintained at 70 °C (ISO/CD 14502-2) (International Standard Organization/Committee Draft Number). Weighed dry leaves were ground well and passed through 30 mesh sieve and used for the analysis of catechin fractions.

Oven dried leaf powder (0.2 g) was extracted using 10.0 ml of 70% methanol on a water bath maintained at 70 °C. One ml of the extract was diluted to 5.0 ml with a stabilizing solution (0.25% each of EDTA and ascorbic acid in 10.0% acetonitrile) according to ISO/CD 14502-2 (International Standard Organization/Committee Draft Number). The sample was filtered through 0.45 μm nylon membrane filter (Millipore, USA) and used for HPLC analysis using Hewlett Packard Model 1100 Series, USA fitted with 5 μm Luna Phenyl-Hexyl bonded column (Phenomenex, USA).

Acetic acid (2.0%) and acetonitrile (9.0%) were used as mobile phase A and acetonitrile (80%) as mobile phase B. The gradient program was as follows: 100% mobile phase A for 10 min, followed by 68% mobile phase A for 15 min and a linear gradient of 32% mobile phase B and a hold at this condition for 10 min. The conditions were then reset to 100% mobile phase A and allowed to equilibrate for 10 min before next injection [28]. Absorbance was measured at 278 nm. The reference standards (Gallic acid (GA), epigallo catechin (EGC), (+) catechin, epicatechin (EC), epigallo catechin gallate (EGCG), caffeine and epicatechin gallate (ECG)) were procured from Sigma–Aldrich (St Louis, MI, USA) and used in spiking tests. Based on the curve integration, the data processing was performed using HP Chem station software (Hewlett Packard, USA).

2.8. Analysis of flavonoid gene expression (by semi-quantitative RT-PCR)

The total RNA extracted from RNeasy Plant Mini Kit (QIAGEN, USA) the segregated two leaves and an apical bud of UPASI-16 and P/11/15 were treated with RNAs- free DNAase I (TaKaRa). DNA free total RNA (100 ng) was used in cDNA synthesis in a reaction volume of 10 μl containing 2.5 units of AMV reverse transcriptase XL (Takara, Japan) and 1 μM of *oligo-dt3sap*. The PCR reaction mixture contained 1.0 μl of template, 2.5 μl of 10× buffer, 0.5 units of Taq polymerase (Fermentas), 1.5 μl of 2.5 mM $MgCl_2$, 1.0 μl of 2.0 mM dNTP and 0.1 mM of 1 μl gene specific primers. The Gene specific primers used were ANS-5′-ATG ACT ACA GTG GCT GCC CCG AGAG-3′; 5′-CTGAGCAAAAGTCCTCGGCGGGAA-3′), F35H-5′-TAGACACCCGTCTTCCTGCTTC GT-3′; 5′GCA GCATAAGCATTGGAGGCAACC-3′), F3H-5′ATGGCGC CACAACGCTTAC-3′; 5′-TCAAGCAAAAATCTCATCAG TC3′) and ANR-5′ATGGAAGCCCAACCGACAGC TC-3′; 5′-TCAATTCTTCAAAATCCCCTTAGCCT-3′). PCR amplification was performed in PTC – 200 thermal cycler (MJ Research, USA) using the following program: initial 94 °C for 2 min followed by 94 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 5 min. The cycle conditions and annealing temperature varied viz., 33 cycles of 58 °C for 45 s (ANS), 27 cycles of 62 °C for 45 s (F35H), 33 cycles of 63 °C for 45 s (F3H) and 33 cycles of 63 °C for 45 s (ANR) respectively.

The PCR products were loaded on 1.5% agarose gel, stained with ethidium bromide visualized under the UV-Transilluminator (Hoefer, Inc, U.S.A) and documented. Reverse and forward primers of *C. sinensis* 26S rRNA (Cs26SrRNA, GenBank accession No. AY283368) were used as internal control. The semi-quantitative PCR assay was performed and analyzed at least twice. The intensity of the bands was quantified using Image J software (http://rsb. info.nih. gov/ij) and normalized against Cs26SrRNA band intensity. The average expression ratio of target gene was calculated between the normalized relative intensity of band in the low and the high PPO from two independent RT-PCR reactions.

2.9. Statistical analysis

Data collected pertaining to PPO activity were subjected to ANOVA by one factor analysis using AGRES software (7.01 versions). The critical difference was noted and significance was determined at $P = 0.05$ levels [29].

3. Results and discussion

3.1. Biochemical analysis

The selected P/11/15 tea accession bound and soluble PPO activity was observed in first Internode (49.62 U/mg of protein) followed by first leaf (47.62 U/mg of protein) second in leaf and bud, third in Leaf. In our early studies a similar high PPO (1178.63 U/mg of protein) activity was observed in the first Internode (23.66 U/mg of protein) followed by first in leaf (22.46 U/mg of protein) second in leaf and bud, and third in UPASI 16 accessions. The total Polyphenol 20.01–17.97% contents were similar in the following order, first Internode > first leaf > second leaf > bud > third leaf. The catechin content ranged from third leaf and bud (13.59% to first Internode (20.75%). The highest peroxidase activity was observed in the first Internode (450.08 μM of O_2 formed $min^{-1}\,g^{-1}$ dry weight) followed by bud (414.78 μM of O_2 formed $min^{-1}g^{-1}$ dry weight) first leaf, second leaf and third leaf. A great increase in the resistance to *P. syringae* was observed in PPO-overexpressing tomato plants; the results indicate PPO-mediated phenolic oxidation in restricting plant disease development [30].

Catalase activity of P/11/15 ranged between 0.47 and 1.20 µM H_2O_2 reduced min^{-1} mg^{-1} protein. In UPASI-16, SOD activity ranged between 16.45 and 45.11 U/mg proteins. In addition, the first Internode and bud showed relatively a higher activity followed by leaves in this clone. PAL activity decreased from bud to third leaf, which ranged from 10.12 to 26.75 µM of cinnamic acid formed per minute (Table 1). The level of SOD, CAT, GPX was increased in V79-4 cells after treating with the extracts of *G. uralensis*, *S. miltiorrhiza*, *P. suffruticosa*, *S. polyrrhiza*, *A. C. var. dulcissima*, *C. officinalis*, *A. officinarum*, *N. nucifera*, and *C. cassia*. In particular, treatment with total extracts of *Areca catechu var. dulcissima* induced higher antioxidant enzyme activities than the other samples [31]. Chilling stress activates the enzymes of an SO D:ascorbate–glutathione cycle under catalase deactivation in the leaves of cucumber, but due to various environmental stresses the response timing of enzyme isoforms is not the same for all isoforms of antioxidant enzymes. Activities of catalase and peroxidase enhanced in chilling stressed-plants as compared to control plants [32]. SOD, GPX, APX, DHAR and GRin salt-stressed leaves induced by Si addition were increased, thus by improving the growth of cucumber plants hence prevent from oxidative damage under salt stress [33].

3.2. Protein profile P/11/15 clone

The protein profile (number and intensity of bands) of P/11/15 clone was compared based on PPO activity at 12% SDS–PAGE. SDS–PAGE analysis revealed more than forty-two protein bands which were present with molecular weight ranging from 19.5 to 213 kDa. Variations in the intensity of protein bands were observed in the range of 15–20 kDa and 30–40 kDa (Fig. 1). Although, the similarities, the intensity of protein bands at 30–40 kDa range in all parts of the crop shoots were almost absent, the absence or less intense protein bands reflect low expression of the corresponding protein [34].

3.3. HPLC analysis of catechin fraction

The catechin fractions of powdered dried tea leaves were analyzed by comparing the retention time of the standard compounds under similar chromatographic conditions [35]. Seven distinct catechin fractions were identified at different time intervals within 30 min. Gallic acid appeared at the 4th minute followed by epigallocatechin (EGC) at 8th minute, caffeine (CA) at 10th minute, simple catechin at 14th minute, epicatechin (EC) at 16th minute, epigallocatechin gallate (EGCG) at 18th minute and epicatechin gallate (ECG) at 25th minute. From the HPLC analysis, it was evident that caffeine, epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were more predominant (Fig. 2 and Table 2). From the HPLC analysis, significant variation in catechin content was observed in the crop shoots and mature leaves. Epicatechin was present as extension units in freeze-dried Lowbush blueberry, cranberry, brown sorghum bran, blueberry, cranberry and Cocoa beans by Normal-phase HPLC analysis [36].

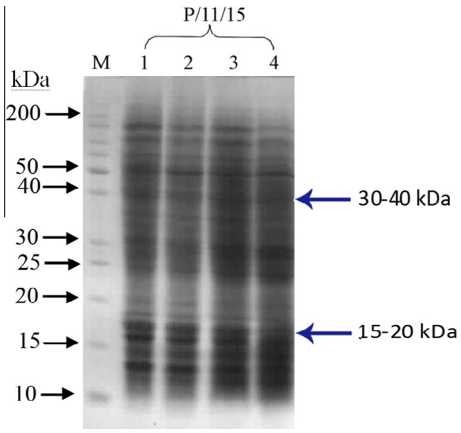

Figure 1 Protein profile of P/11/15 clone. Lane: M – Marker; 1 – Bud; Lane 2 – first Internode; Lane 3 – first Leaf; Lane 4 – second Leaf.

3.4. Flavonoid gene expression by semi-quantitative RT-PCR

Total RNA was isolated from the bud of P/11/15 clone. Two distinct bands for 18S and 28S were observed. The $A_{260/280}$ ratio was 1.8–2.0. The expression of flavonoid genes viz., ANR, F3H, F35H and ANS in the segregated crop shoot (bud, first internode, first leaf and second leaf) of selected accession of P/11/15 were revealed by semi quantitative RT-PCR. The 26S rRNA gene was used as a reference to analyze the cDNA that were preferentially expressed in the segregated samples. Amplification was efficient with 50 ng ml^{-1} of total RNA in all part of the shoots. Higher expression of F35H

Table 1 Distribution of enzymes and substrates in P/11/15 clone.

Biochemical parameter	Bud	First leaf	First internode	Second leaf + Bud	Third leaf + Bud	SEM ±	C.D. @ $P = 0.05$
Polyphenol Oxidase[a]	39.43	47.62	49.62	39.79	32.96	7.65	17.05
Peroxidase[b]	414.78	350.08	450.08	316.74	240.04	34.59	77.08
Superoxidase dimutase[c]	34.78	27.44	45.11	22.11	16.45	1.79	3.99
Phenylalanine ammonia lyase[d]	26.75	19.33	15.55	16.85	10.12	0.41	0.92
Catelase[e]	1.06	0.99	1.20	0.77	0.47	0.10	0.23
Polyphenols (%)	18.22	19.99	20.01	19.05	17.97	0.28	0.63
Catechin (%)	14.35	19.29	20.75	17.38	13.59	0.29	0.63

[a]U/mg of protein, [b]µmole of O_2 formed min^{-1} g^{-1} dry weight, [c]U/mg of protein, [d]µmol of cinnamic acid formed min^{-1} g^{-1} acetone powder, [e]µmol H_2O_2 reduced min^{-1} mg^{-1} protein, polyphenol, catechin in percentage. Each value is the mean of triplicate. SEM-Standard Error Mean. C.D.-Critical difference.

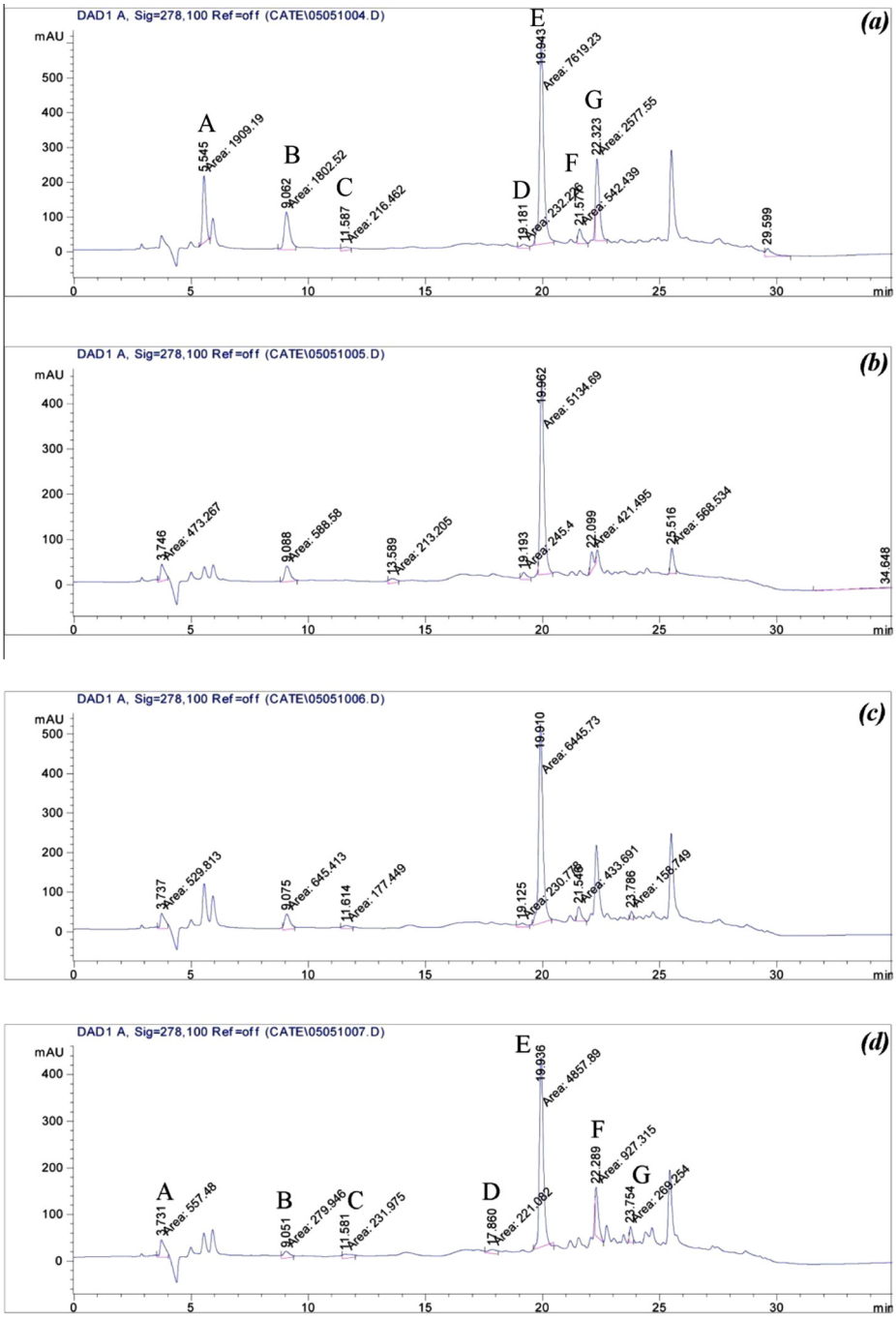

Figure 2 HPLC chromatogram of catechin fractions in P/11/15 clone. (a) Bud, (b) first internode, (c) first leaf and (d) second leaf. A: gallic acid (GC); B: epigallocatechin (EGC); C: simple catechin (SC); D: caffeine; E: epicatechin (EC); F: epigallocatechin gallate (EGCG); G: epicatechin gallate (ECG).

was observed in the bud of P/11/15 (0.57%) followed by the first internode, first leaf and second leaf. A similar pattern was observed with the rest of the Flavonoid gene expression (bud > first internode > first leaf > second leaf). Higher F3H (1.02%) and ANR (1.66) expression was observed in P/11/15 clone (Table 3 and Fig. 3).

To understand the gene regulatory network of catechin biosynthesis, it is essential to study the differentially expressed genes in leaves at various developmental stages. The enzymes such as F3H, F35H, ANR, DFR, and LCR were found cross linking between phenylpropanoid and flavonoid pathways during the biosynthesis of catechin. Genes related to catechin biosynthesis has been cloned and the relationship between expressions of these genes in the accumulation of catechins was observed by Kashmir singh et al. [36] reported that the concentration of catechins and F3H expression in leaves of different developmental stages in tea (*C. sinensis* (L.) O. Kuntze) leaves a positively correlated.

Table 2 Relative distribution of catechin fractions in P/11/15 clone.

Fraction No.	Catechin fractions (%)	Bud	First leaf	First internode	Second leaf	Third leaf	SEM ±	C.D.@ $P = 0.05$
1	EGC	2.85	3.58	3.99	3.21	2.58	0.03	0.07
2	SC	0.74	0.98	1.23	0.75	0.69	0.02	0.04
3	EC	1.29	1.85	2.2	1.32	0.99	0.04	0.09
4	EGCG	8.62	10.25	11.01	9.65	6.69	0.05	0.11
5	ECG	1.41	2.85	2.85	1.58	1.02	0.06	0.10
6	TC	13.9	19.51	21.28	16.5	12.99	0.14	0.31

EGC – epigallocatechin, SC – simple catechin, EC – epicatechin, EGCG – epicatechin gallate, ECG – epicatechin gallate and TC – total catechin. SEM-Standard Error Mean. C.D.-Critical difference.

Table 3 Semi quantitative RT-PCR analysis of flavonoid gene in P/11/15 clone.

Gene	Bud	First internode	First leaf	Second leaf
F35H	0.57	0.42	0.17	0.02
ANS	2.58	2.53	2.46	2.13
F3H	1.02	0.39	0.25	0.06
ANR	1.66	0.99	0.76	0.36
26S rRNA	1.00	1.00	1.00	1.00

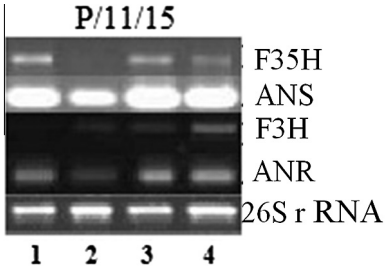

Figure 3 Flavonoid gene expression in P/11/15 clone. Lane: 1 – bud, 2 – first internode, 3 – first leaf, 4 – second leaf.

4. Conclusion

In the present study selected clone of *C. sinensis* (L) O. Kuntze has comparable PPO enzyme activity, on the other hand it has sufficient enzyme for antioxidant activity (Polyphenol, peroxidise, Catalase, SOD, PAL). The HPLC analysis also confirmed that, the selected clone of P/11/15 has adequate catechin in the extract. Through the gene expression study, a higher F3H (1.02%) and ANR (1.66) gene expression was observed in P/11/15 clone. Based on the results of this study it can be concluded that, ethyl alcohol extract of *C. sinensis* (L) O. Kuntze from P/11/15 clone has sufficient biochemical enzymes responsible for antioxidant activity.

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